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A Widespread Glutamine-Sensing Mechanism in the Plant Kingdom

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SUMMARY

Glutamine is the primary metabolite of nitrogen assimilation from inorganic nitrogen sources in microorganisms and plants. The ability to monitor cellular nitrogen status is pivotal for maintaining metabolic homeostasis and sustaining growth. The present study identifies a glutamine-sensing mechanism common in the entire plant kingdom except *Brassicaceae*. The plastid-localized P_{II} signaling protein controls, in a glutamine-dependent manner, the key enzyme of the ornithine synthesis pathway, *N*-acetyl-L-glutamate kinase (NAGK), that leads to arginine and polyamine formation. Crystal structures reveal that the plant-specific C-terminal extension of P_{II}, which we term the Q loop, forms a low-affinity glutamine-binding site. Glutamine binding alters P_{II} conformation, promoting interaction and activation of NAGK. The binding motif is highly conserved in plants except *Brassicaceae*. A functional Q loop restores glutamine sensing in a recombinant *Arabidopsis thaliana* P_{II} protein, demonstrating the modular concept of the glutamine-sensing mechanism adopted by P_{II} proteins during the evolution of plant chloroplasts.

INTRODUCTION

Glutamine is the primary product of nitrogen assimilation from inorganic nitrogen sources and a central metabolite in nitrogen metabolism in plants. The regulation of nitrogen metabolism and the maintenance of a proper carbon-nitrogen balance in plants are extremely complex and interwoven at multiple levels. How plants sense nitrogen status is an intriguing issue of current research (e.g., Nunes-Nesi et al., 2010). Glutamine is an important nitrogen status reporter in many bacteria (Forchhammer, 2007) but, in plants, glutamine sensing is poorly understood. In prokaryotes, P_{II} signaling proteins (in the following termed P_{II} proteins) have been identified as central signal-integrating molecules coordinating nitrogen assimilatory reactions in response to the general metabolic state of cells. The P_{II} protein family represents one of the most abundant families of signaling proteins in nature, occurring in bacteria, Archaea, and plants (Chellamuthu et al., 2013; Leigh and Dodsworth, 2007; Sant'Anna et al., 2009). P_{II} proteins are able to sense and integrate signals from central metabolism, in particular 2-oxoglutarate (2-OG), an indicator of C-N balance, as well as the energy status via competitive ADP/ATP binding (Fokina et al., 2010; Jiang and Ninfa, 2009b; Zeth et al., 2014).

The concentration of these metabolite effectors is sensed by binding to an ensemble of intercommunicating sites in the trimeric P_{II} proteins, where the actual effector molecule binding status is translated into distinct P_{II} conformations. The T loop, a highly flexible loop segment protruding from each subunit, plays a key role in ligand binding and receptor interactions (Forchhammer, 2008; Huergo et al., 2013). Depending on the conformation of the T loop, P_{II} proteins can bind to various receptors, thereby exerting control at all levels of metabolic regulation (transport activity, metabolic reactions, gene expression). This basic mode of P_{II} function, based on direct sensing of effector molecules, is conserved in almost all P_{II} proteins.

A second, phylogenetically diverse regulatory layer may be superposed on P_{II} signaling: covalent modification of apical residues of the T loop allows the integration of additional signals. In proteobacteria, P_{II} proteins can be covalently modified by uridylylation at T loop residue Tyr51. The modifying enzyme GlnD, a bifunctional uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59), responds to the cellular glutamine level such that low glutamine levels trigger the P_{II}-uridylylation reaction (Adler et al., 1975; Ninfa and Jiang, 2005). This results in Tyr51-uridylylated P_{II}, which is, for instance, not able to interact with the ammonium channel AmtB (Radchenko and Merrick, 2011). Conversely, increasing glutamine levels cause P_{II}-deuridylylation (Adler et al., 1975), allowing the interaction of P_{II} with various targets (Huergo et al., 2013). As a consequence, glutamine-dependent uridylylation/deuridylylation of P_{II} is the prevailing signaling mechanism of the *Escherichia coli* P_{II} system (Forchhammer, 2007; Ninfa and Atkinson, 2000). In analogy to proteobacterial P_{II}-uridylylation, P_{II} in actinobacteria is adenylylated at Tyr51 in a glutamine-dependent manner (Hesketh et al., 2002). In cyanobacteria, the neighboring residue Ser49 is phosphorylated in response to nitrogen starvation (Forchhammer and Tandeau de Marsac, 1994), but the detailed molecular signals controlling the kinase activity are still unknown. In many other organisms, this second regulatory layer of covalent modification of the T

loop is apparently missing, as in *Bacillus* (Heinrich et al., 2006), Archaea (Leigh and Dodsworth, 2007), and plant P_{II} proteins (Smith et al., 2004; Uhrig et al., 2009).

Phylogenetic analysis has inferred that plant P_{II} proteins are of cyanobacterial origin and have been conserved during the evolution of the *Chloroplastida* (plant kingdom, comprising green algae and land plants) from the ancestral cyanobacterial endosymbiont to higher plants. By contrast, P_{II} signaling has been lost in some red algae and in the *Chromalveolata* (Chellamuthu et al., 2013). The only plant P_{II} protein that has been studied biochemically in depth is that of *Arabidopsis thaliana* (Mizuno et al., 2007a; Smith et al., 2003). Physiological studies indicate that plant P_{II} proteins are part of a complex signal-transduction network mediating nitrogen regulation (Hsieh et al., 1998; Nunes-Nesi et al., 2010). P_{II} mutants of *A. thaliana* are affected in the synthesis of compounds derived from the ornithine/arginine synthesis pathway (Ferrario-Méry et al., 2006), overaccumulate carbon metabolites (Ferrario-Méry et al., 2005), and show impaired control of nitrite uptake into the chloroplast (Ferrario-Méry et al., 2008). Furthermore, P_{II} seems to be required for proper seed development (Uhrig et al., 2009). Such tissue-specific roles of P_{II} signaling together with the tightly intertwined complex network of signal-transduction cascades (Nunes-Nesi et al., 2010) complicate the elucidation of P_{II}-specific functions in higher plants. To study fundamental problems in plant chloroplasts, the simple unicellular green alga *Chlamydomonas reinhardtii* has proved an excellent model organism (Harris, 2001). For this reason, we started studying P_{II} signaling in *C. reinhardtii* (Ermilova et al., 2013).

Like its higher plant homologs, *C. reinhardtii* P_{II} (*Cr*P_{II}) is localized in the chloroplast. Residues involved in effector molecule binding are conserved, which suggests that *Cr*P_{II} functions as a signaling protein as well. The chloroplast-localized enzyme *N*-acetyl-L-glutamate kinase (NAGK), which catalyzes the committed step in the ornithine/arginine biosynthesis pathway, is an important P_{II}-interacting protein in cyanobacteria and higher plants (Ferrario-Méry et al., 2006; Heinrich et al., 2004; Sugiyama et al., 2004). The structures of the P_{II}-NAGK complex from the cyanobacterium *Synechococcus elongatus* and from *A. thaliana* have been solved and are highly similar (Llácer et al., 2007; Mizuno et al., 2007b). Beez et al. (2009) have shown that the P_{II}-NAGK proteins from *S. elongatus* and *A. thaliana* functionally complement each other in vitro, highlighting striking functional conservation. Binding of P_{II} enhances NAGK activity in the presence of the feedback inhibitor arginine, an effect that can be antagonized by the P_{II}-effector molecule 2-OG. When P_{II} binds to NAGK, the T loops of P_{II} must fold in a tightly flexed structure to insert into the interdomain crevice of the adjacent NAGK subunits (Llácer et al., 2007; Mizuno et al., 2007b).

An unsolved question in the evolution of plant P_{II} proteins concerns the appearance of an elongated C terminus. Whereas all bacterial P_{II} proteins have an almost invariable C terminus, the P_{II} proteins from the plant kingdom contain a C-terminal extension of 13–19 amino acids with a conserved motif. The function of the C-terminal extension is so far unknown. In the present work, we studied the structural and biochemical properties of *Cr*P_{II}. We discovered that the C-terminal extension of *Cr*P_{II} forms a small loop structure (termed the Q loop) that binds glutamine and is required for glutamine-dependent complex formation with NAGK. The same glutamine dependence was observed for recombinant *Physcomitrella* and *Oryza* P_{II} proteins, which implies that plant P_{II} proteins function as glutamine sensors via the C-terminal Q loop extension.

RESULTS

Basic Properties of *C. reinhardtii* NAGK, the Putative P_{II} Target

The predicted full-length NAGK polypeptide encoded by the *C. reinhardtii AGK1* cDNA consists of 340 amino acids with a calculated molecular mass of 35,971 Da. The N terminus contains a predicted chloroplast transit peptide (residues 1–42), which suggests that, like its higher plant homologs, *C. reinhardtii* NAGK (*Cr*NAGK) protein resides in the chloroplast with a molecular mass of 31,474 Da. A previous survey showed that all NAGK proteins from cyanobacteria and plants (*Chloroplastida*), and from those red algae that contain P_{II} proteins, share the signature residues involved in NAGK-P_{II} interaction (Chellamuthu et al., 2013; an alignment is shown in Figure S1 available online), which strongly implies that they all are targets of P_{II} signaling. To study the biochemical properties and to characterize the putative interaction between *Cr*P_{II} and *Cr*NAGK, a recombinant *Cr*NAGK protein was generated (starting from residue Met44) with its N terminus fused to a His_6 tag. Analysis of the purified *Cr*NAGK protein by gel filtration revealed that most of the protein eluted in the expected hexameric state, whereas a smaller fraction eluted with an apparent higher mass. By comparison, *A. thaliana* NAGK (*At*NAGK) eluted as a single peak, corresponding to the hexameric enzyme (Figure S2). The kinetic constants of *Cr*NAGK showed a K_m value for *N*-acetyl glutamate of 7.77 mM and a k_{cat} of 56.76 s^{-1} (calculated with NAGK hexamer). This K_m value is 9-fold higher than that of *At*NAGK but very similar to that of the cyanobacterial *S. elongatus* NAGK (*Se*NAGK) (K_m [NAG] of 7.4 mM), whereas the k_{cat} value is intermediate between that of *At*NAGK (126 s^{-1}) and *Se*NAGK (13 s^{-1}). Feedback inhibition by arginine occurred with a half-maximal inhibitory concentration (IC_{50}) of 0.11 mM (Figure 1B), which is intermediate between that of cyanobacterial *Se*NAGK (0.02 mM) and plant *At*NAGK (1 mM).

Glutamine-Dependent Interaction between *Cr*P_{II} and NAGK

To test the effect of *Cr*P_{II} on the catalytic activity of *Cr*NAGK, we assayed NAGK using a coupled ATPase assay (Beez et al., 2009). In a control, ATPase activity of *Cr*P_{II} in the absence of NAGK was below the detection limit of the assay. The previously reported ATP hydrolysis activity of P_{II} proteins is not catalytic, but needs a large excess of P_{II} over ATP (Radchenko et al., 2013).

Determining the relief from arginine inhibition by P_{II}-NAGK complex formation represents the most sensitive assay for P_{II}-NAGK interaction. In arginine-inhibition assays with *Cr*NAGK in the presence or absence of *Cr*P_{II} (Figure 1A), the presence of *Cr*P_{II} surprisingly did not change arginine inhibition of *Cr*NAGK, nor did it change its catalytic activity. As a possible suitable positive control of *Cr*P_{II} function, we tested the effect of *Cr*P_{II} on *At*NAGK, which we previously showed to functionally interact

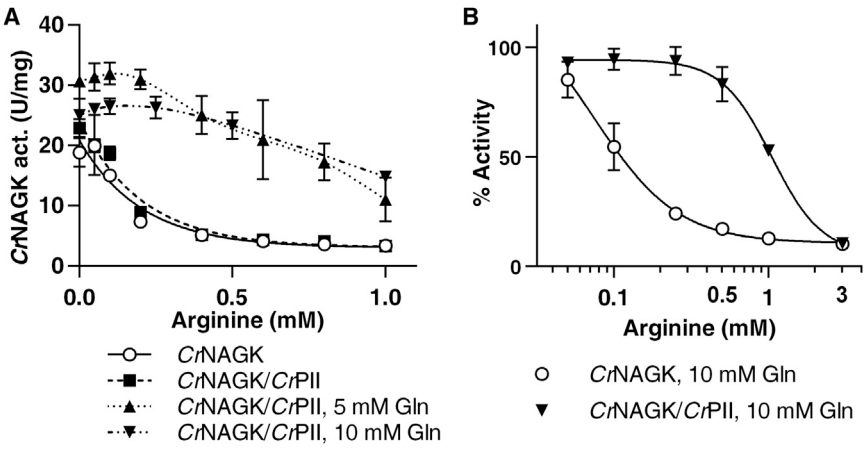

Figure 1. Arginine Inhibition of *Cr*NAGK Activity in the Presence of CrP_{II}

(A) Arginine inhibition of *Cr*NAGK in the absence or presence of CrP_{II}, without or with 5 or 10 mM glutamine, as indicated.

(B) Arginine inhibition of *Cr*NAGK in the presence of 10 mM glutamine or in the presence of CrP_{II} and 10 mM glutamine. Data were fitted to a sigmoidal dose-response curve, yielding an EC_{50} (arginine) of 0.11 ± 0.04 mM and 1.05 ± 0.15 mM for free or CrP_{II}-interacting *Cr*NAGK, respectively.

When exceeding the size of the data point symbols, standard deviation from triplicate measurements is indicated by error bars.

not only with its own P_{II} protein but also with P_{II} from the cyanobacterium *S. elongatus* (Beez et al., 2009). However, CrP_{II} did not affect the arginine response of *At*NAGK (data not shown). We therefore suspected that an additional component may be required for CrP_{II} interaction with NAGK. We added amino acids and metabolites from core metabolism (glutamate, glutamine, aspartate, asparagine, alanine, serine, glycine, acetyl-CoA, and tricarboxylic acid cycle intermediates) to the arginine-inhibition assays of *Cr*NAGK-CrP_{II}. Of the various compounds tested, glutamine indeed rescued the activity of CrP_{II}; in the presence of 5 or 10 mM glutamine, the inhibitory effect of arginine was strongly antagonized (Figure 1A). In the presence of P_{II} and 10 mM glutamine, the IC_{50} of arginine for *Cr*NAGK increased from 0.1 mM to 1 mM (Figure 1B). The highest difference in activity between *Cr*NAGK with or without CrP_{II} was with 0.4 mM arginine. Therefore, we titrated the effect of glutamine with 0.4 mM arginine. Glutamine activated arginine-inhibited *Cr*NAGK in the presence of CrP_{II} in a concentration-dependent manner (Figure 2A), whereas glutamine had no effect in the absence of CrP_{II}. The half-maximal effective concentration (EC_{50}) of glutamine for activation of NAGK by CrP_{II} was 2.4 ± 0.8 mM. To reveal whether the glutamine dependence of CrP_{II}-NAGK activation is a property of CrP_{II} or of *Cr*NAGK, heterologous assays were carried out using *At*NAGK. CrP_{II} had no effect on *At*NAGK in the absence of glutamine. By contrast, arginine inhibition was relieved by glutamine in a concentration-dependent manner (Figure 2B). The response was similar to the glutamine-dependent activation of *Cr*NAGK by CrP_{II}, with a glutamine EC_{50} of 4.6 ± 2.4 mM, which showed that the glutamine dependence is a property of CrP_{II}.

To determine the response of CrP_{II}-*Cr*NAGK interaction toward 2-OG, we titrated CrP_{II} and *Cr*NAGK (5 CrP_{II} trimers:1 *Cr*NAGK hexamer) in the presence of 10 mM glutamine and 0.4 mM arginine with increasing concentrations of 2-OG. 2-OG tuned down NAGK activity with an IC_{50} of 1.18 ± 0.02 mM (Figure 2C). An almost identical response toward 2-OG was obtained for the heterologous assay with CrP_{II} and *At*NAGK (Figure 2D; IC_{50} of 1.26 ± 0.96 mM). The catalytic constants of the CrP_{II}-*Cr*NAGK complex determined at 5 mM glutamine showed that CrP_{II} does not enhance the V_{max} (54 s^{-1}) of the NAGK reaction but lowers the K_m for NAG about 2-fold (3.9 ± 0.4 mM as compared to 7.8 ± 0.8 mM).

Analysis of CrP_{II}-NAGK Complex Formation

To confirm that the glutamine-dependent regulatory effect of CrP_{II} on NAGK is due to direct interaction, we analyzed complex formation using surface plasmon resonance (SPR) spectroscopy. The N-terminally His-tagged *Cr*NAGK protein was immobilized on an Ni-NTA sensor chip and probed with CrP_{II} together with effector molecules. CrP_{II} bound to *Cr*NAGK in a glutamine-dependent manner; with 5 mM glutamine, complex formation appeared to be saturated (Figure 3A). This result confirmed that complex formation between CrP_{II} and *Cr*NAGK is strictly glutamine dependent. Furthermore, complex formation of CrP_{II} with *Cr*NAGK strictly required Mg-ATP and was not supported by ADP (Figure 3B). Finally, we analyzed the effect of 2-OG on CrP_{II}-NAGK complex formation. Regardless of the 2-OG concentration added to the assay mixture (containing 1 μM CrP_{II} and 5 mM glutamine), no antagonistic effect on complex formation was observed (Figure 3C), although these 2-OG concentrations prevented activation of NAGK (Figure 2C). This result resembles the case of *A. thaliana* P_{II}-NAGK interaction, in which the 2-OG concentrations required to inhibit complex formation are three orders of magnitude higher than those required to impair NAGK activation by P_{II} (Beez et al., 2009). Similarly, in *E. coli*, the regulation of NtrB activity by P_{II}, but not its binding, is controlled by 2-OG (Jiang and Ninfa, 2009a). Thus, the inhibitory effect of 2-OG on P_{II}-mediated activation of NAGK in *C. reinhardtii* appears to occur postbinding.

Structural Characterization of CrP_{II}

The EC_{50} of glutamine for stimulating CrP_{II}-NAGK interaction was in the millimolar range, which indicated a low affinity of CrP_{II} for glutamine, outside the sensitivity range of direct ligand-binding assays. Titration of glutamine to CrP_{II} by isothermal titration calorimetry (ITC) yielded only marginal calorimetric signals. To define the role of glutamine and identify its putative binding site in CrP_{II}, we used crystallographic analysis. First, we set up CrP_{II} crystallization trials in the presence of different effector molecules. From these trials, we obtained well-diffracting crystals that grew in the presence of Mg-ATP and glutamine, for which we could solve the structure by molecular replacement using the SeP_{II} structure (Protein Data Bank [PDB] ID code) 2XZW as a search model. The resulting CrP_{II} structure has the expected

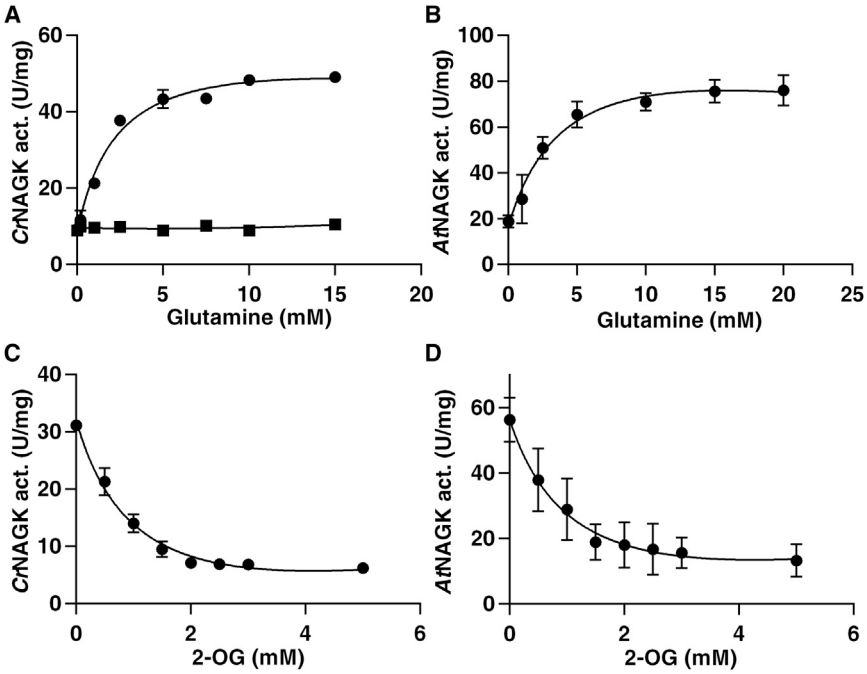

Figure 2. Effect of Glutamine or 2-OG on *Cr*P_{II} Activation of *Cr*NAGK and *At*NAGK

*Cr*NAGK assays contained 0.4 mM arginine; *At*NAGK assays contained 3 mM arginine.

(A) Glutamine-dependent activation of *Cr*NAGK by *Cr*P_{II} (circles) or by *Cr*P_{II}-ΔC (squares).

(B) Glutamine-dependent activation of *At*NAGK by *Cr*P_{II}.

(C and D) Antagonistic effect of 2-OG on *Cr*P_{II}/10 mM glutamine-mediated activation of (C) *Cr*NAGK or (D) *At*NAGK.

The standard deviation from triplicate measurements is indicated by error bars.

trimeric assembly and superimposes (with a root-mean-square deviation [rmsd] of 0.85 Å over 271 aligned Cα positions) closely on the *Se*P_{II} structure 2XZW. However, neither Mg-ATP nor glutamine was identified in the structure, and both the T loop and the whole C-terminal extension were disordered (Figure 4A). Therefore, we then tried to bind ligands to the protein by soaking the apo crystals in solutions containing different combinations of effector molecules. This was successful for the combination of Mg-ATP and 2-OG, which resulted in a crystal structure with asymmetrically occupied effector sites—one site occupied by Mg-ATP and 2-OG in full occupancy, the second site occupied by Mg-ATP and 2-OG in half-occupancy, and the third site empty (Figure 4B). Half-occupancy denotes that, on average, throughout the crystal, only every second P_{II} trimer has a molecule bound in the respective binding site, which indicates an interaction weaker than binding with full occupancy. The structure superimposes with an rmsd of 0.47 Å on the apo structure, and both the T loop and C-terminal extension are unstructured in all subunits. The binding mode of the effector molecules is essentially the same as reported for various bacterial and archaeal P_{II} proteins (Fokina et al., 2010; Maier et al., 2011; Truan et al., 2010). The asymmetrical filling of the sites suggests that binding of the effector molecules is anticooperative, as reported previously for the *E. coli* (Jiang and Ninfa, 2009a) and *S. elongatus* P_{II} protein (Fokina et al., 2010; Zeth et al., 2014).

Identification of the Glutamine-Binding Site in the P_{II}-NAGK Complex Structure

Crystallization trials were carried out with *Cr*P_{II}-NAGK complexes in the presence of glutamine and different combinations of effector molecules. Because initial crystallization trials with the homologous *Cr*NAGK were not fruitful, we set up trials with the heterologous *At*NAGK, because the glutamine response of the *Cr*P_{II}-*At*NAGK complex was almost identical to that of the homologous *Cr*P_{II}-*Cr*NAGK complex. This setup rewarded us with crystals of the whole complex that finally disclosed the binding mode of glutamine. Glutamine was localized to the C-terminal extension of *Cr*P_{II}. Interestingly, these *Cr*P_{II}-*At*NAGK crystals have the same crystal packing as those of the *At*P_{II}-*At*NAGK structure (PDB ID code) 2RD5, so that the structure could be solved directly on the basis of the 2RD5 coordinates (see Extended Experimental Procedures). Consequently, the complex is almost identical to the *At*P_{II}-*At*NAGK complex and very similar to the *Se*P_{II}-NAGK complex, with a hexameric NAGK toroid sandwiched between two P_{II} trimers (Figure 5A). As described for the *A. thaliana* P_{II}-NAGK complex (Mizuno et al., 2007b), the NAGK subunits are in an open conformation on one face of the toroid and in a closed conformation on the other face. The ligand-binding sites of the closed subunits are occupied with arginine, NAG, and Mg-ADP, whereas the subunits in open conformation are occupied with arginine and the reaction product NAG-phosphate but not with a nucleotide. P_{II} is in complex with the desired glutamine and Mg-ATP, although no ATP had been added to the crystallization solution (Figure 5B). The presence of ATP, presumably originating from a contamination, further strengthens the conclusion that *Cr*P_{II} can only bind *At*NAGK in the Mg-ATP complex.

Because most residues important for complex formation are conserved between *Cr*P_{II} and *At*P_{II}, it is not surprising that the overall structure and in particular the conformation of the T loop of the *Cr*P_{II}-*At*NAGK and *At*P_{II}-*At*NAGK (2RD5) complexes are nearly identical. A superposition of P_{II} trimers from both complexes yields an rmsd of 0.79 Å over 372 aligned Cα positions, which supports the significance of the *Cr*P_{II} conformation in the heterologous complex.

The remarkable and important difference between *At*P_{II} and *Cr*P_{II} in complex with NAGK concerns the C-terminal extension. In glutamine-bound *Cr*P_{II}, the extension forms two additional short α-helical segments connected by a small loop, from Lys131 to Gly135 (Figure 5C). This extension constitutes a large portion of the glutamine-binding site. Glutamine mainly forms hydrogen bonds to main-chain atoms of this extension and to Arg43, Gly44, and Val82 (Figure 5D). Additional hydrogen bonds

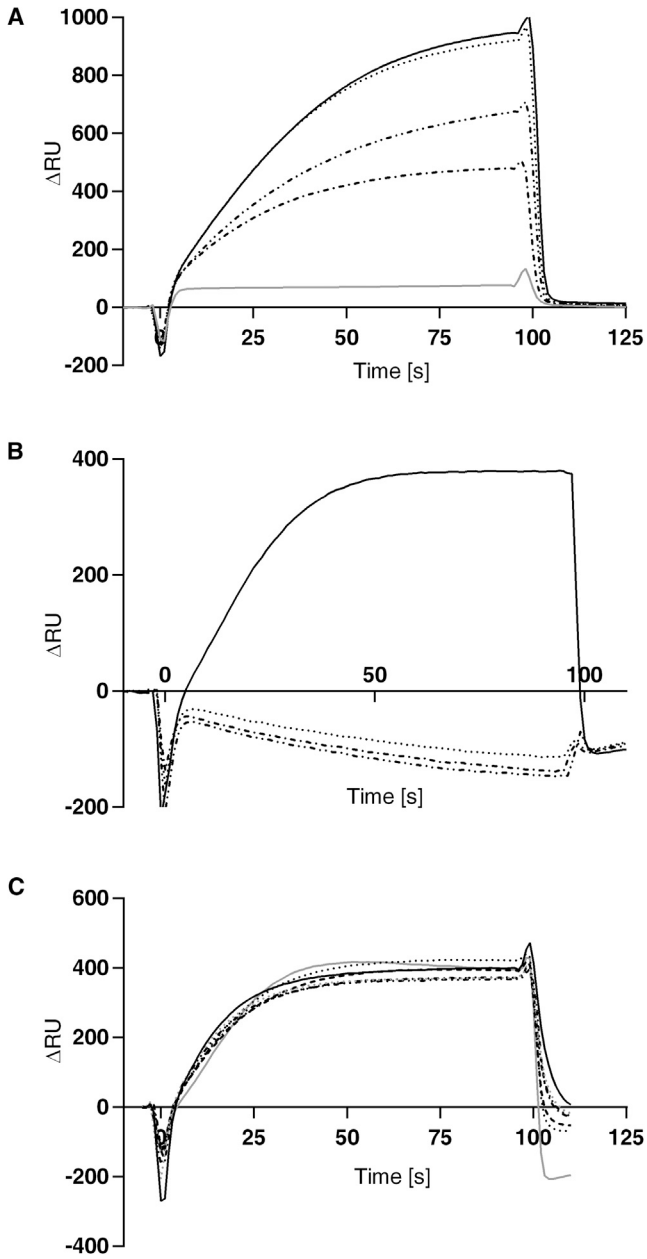

Figure 3. *Cr*P$_{II}$-*Cr*NAGK Complex Formation Analyzed by SPR Spectroscopy

*Cr*P$_{II}$ with effectors was injected on immobilized *Cr*NAGK in flow cell 2; the response difference between FC2 and the control FC1 is shown.

(A) Glutamine-dependent binding of *Cr*P$_{II}$ to *Cr*NAGK at 0 (gray line), 1 mM (dot-dashed line), 2.5 mM (dot-dot-dashed line), 5 mM (dotted line), and 7.5 mM (black line) glutamine.

(B) ATP-dependent complex formation at 5 mM glutamine. Lines: black, 1 mM ATP/2 mM $MgCl_2$; dotted, 1 mM ADP; dash-dotted, 1 mM ADP/2 mM $MgCl_2$; dot-dot-dashed, 1 mM ADP/2 mM $MgCl_2$/5 mM glutamine.

(C) Influence of 2-OG on *Cr*P$_{II}$-*Cr*NAGK interaction at 5 mM glutamine. Gray line, no 2-OG; other lines, 0.5, 1, 3, 5, 10, or 20 mM 2-OG.

are formed with the side chains of Glu130 and Gln87. The structure of the unique C-terminal loop (from Lys131 to Gly135), which we term the Q loop, is further stabilized by contacts with a region from the basal part of the T loop, which also takes part in effector molecule binding (Figure 6A). Two hydrogen bonds are formed between the Q loop and T loop residues Gln56 and Gly57. Of particular interest is Gln56 (corresponding to Gln39 in bacterial P$_{II}$ proteins), as it is a key residue for the structure of the T loop, switching between different P$_{II}$ structural states (Huergo et al., 2013; Zeth et al., 2014). It seems therefore that its interactions with the Q loop help to bring the T loop into its flexed conformation, required for complex formation with NAGK.

Functional Implications of Glutamine Binding

Although the crystal structure reveals the binding site of glutamine, it does not mechanistically explain why glutamine is essential for *Cr*P$_{II}$ complex formation. Either the C-terminal segment acts as an antideterminant, preventing binding to NAGK, or the Q loop in the glutamine-bound state supports complex formation. To distinguish between these possibilities, the C-terminal segment of *Cr*P$_{II}$ was deleted, shortening the protein to the canonical length of bacterial P$_{II}$ proteins. The recombinant truncated *Cr*P$_{II}$ protein (*Cr*P$_{II}$-ΔC) has the expected size of a trimeric P$_{II}$ protein (Figures S3A and S3B). Furthermore, the truncated protein retained the core function of P$_{II}$ proteins, as deduced from 2-OG-binding assays using ITC. The isotherms could be fitted to a one-site binding model with a K_d of 90 μM, as compared to 39 μM for the full-length protein (Figures S3C and S3D). Interaction of *Cr*P$_{II}$-ΔC with NAGK was tested in enzyme assays (Figure 2A) as well as in SPR experiments (Figure S3E). In no assays was interaction detected, neither in the presence nor absence of glutamine, which indicated that the glutamine-binding C-terminal segment is actively required for *Cr*P$_{II}$ to bind to NAGK in a glutamine-dependent manner.

Glutamine Sensing Is a General Property of Plant P$_{II}$ Proteins

Sequence alignment of plant P$_{II}$ proteins (Figure 6C; an extended list is in Figure S4) reveals that the C-terminal residues of the Q loop are part of a conserved motif present in all plant sequences that we analyzed, except the *Brassicaceae* family, to which *A. thaliana* belongs. The Q loop in members of the *Brassicaceae* family exhibits a deletion of three amino acids, which might help the Q loop to fold in a glutamine-independent manner (Figure 6B). To find out whether the missing glutamine response of *A. thaliana* P$_{II}$ is a consequence of this genetic trait, we exchanged the 19 amino acid Q loop extension of *Cr*P$_{II}$ (see Figure 6C) with the 15 amino acid C terminus of *At*P$_{II}$, and vice versa, to obtain chimeric *Cr*P$_{II}$ with the *A. thaliana* C terminus (*Cr*P$_{II}$/*At*Q) and chimeric *At*P$_{II}$ with the *C. reinhardtii* Q loop terminus (*At*P$_{II}$/*Cr*Q). The *Cr*P$_{II}$/*At*Q chimera only very weakly activated *Cr*NAGK (Figure 7A), which indicated that the C terminus of *A. thaliana* P$_{II}$ cannot efficiently replace that of *C. reinhardtii*, and that this replacement completely abrogated the glutamine response. Conversely, the *At*P$_{II}$/*Cr*Q chimera was functional and, moreover, showed partial glutamine dependence (Figure 7B). Therefore, the inability of *A. thaliana* P$_{II}$ to respond to glutamine was indeed caused by the degenerated C terminus. The conserved

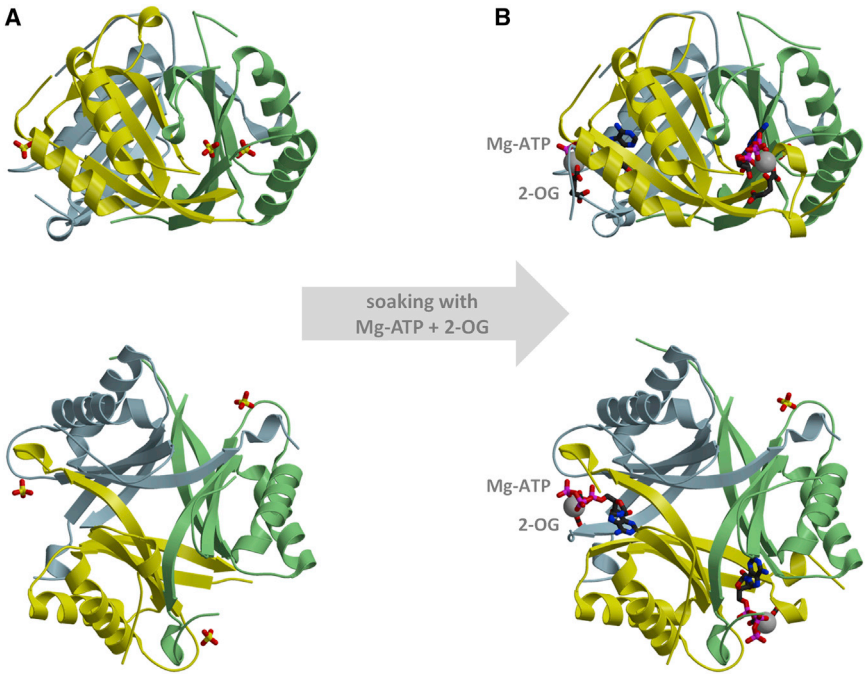

Figure 4. Side and Top Views of the Trimer Structure of *Cr*P_{II} in the Apo State and with Bound Mg-ATP and 2-OG

In the apo structure (A), sulfate ions from the reservoir solution are bound in place of the ATP γ-phosphate in all three monomers. Upon soaking these crystals with Mg-ATP and 2-OG (B), Mg-ATP is bound with full occupancy in two monomers; 2-OG is bound fully in one monomer, with half-occupancy in the other. The third monomer remains in the sulfate-bound apo state. The T loop and the C-terminal extension including the Q loop are unstructured in all states. Data collection and refinement statistics for the structures are shown in Table S1.

Q loop C terminus of plant P_{II} proteins not belonging to the *Brassicaceae* strongly implies that glutamine sensing might be a general feature of P_{II} function in plants. To test this assumption, we prepared recombinant P_{II} proteins from two very diverse plants, the moss *Physcomitrella patens* and the rice *Oryza sativa*. To test the glutamine response, these P_{II} proteins were added to arginine-feedback-inhibited *At*NAGK with increasing concentrations of glutamine. Both P_{II} proteins were able to release *At*NAGK from arginine-feedback inhibition in a glutamine-dependent manner: *Physcomitrella* P_{II} with an EC_{50} of 6.6 mM, and *Oryza* P_{II} with an EC_{50} of 9.2 mM (Figure 7C). This glutamine response is similar to that of *Cr*P_{II} (Figure 2B), which confirmed that glutamine sensing is a general property of plant P_{II} proteins.

DISCUSSION

Maintenance of a proper carbon-nitrogen balance is pivotal for plant growth and development. A functional understanding of the underlying regulatory mechanisms is fundamental to improvement of crop yield and resistance (Nunes-Nesi et al., 2010; Lea and Miflin, 2010). In higher plants, the signal-transduction network associated with nitrogen assimilation is extremely complex, integrating signals from hormones, nitrate, sugars, organic acids, and amino acids (Foyer et al., 2003; Nunes-Nesi et al., 2010; Zheng, 2009). Glutamine, as the primary product of ammonium assimilation, is of particular importance, and is one of the most abundant amino acids of light-grown plants (Fritz et al., 2006). Ammonium is provided by nitrate reduction, photorespiration, and amino acid recycling, and is mainly assimilated in the chloroplast by glutamine synthetase (Keys, 2006; Lea and Miflin, 2010). Glutamine donates nitrogen groups, directly or via glutamate, for essentially all nitrogenous cell compounds.

The present study identified plant P_{II} proteins as glutamine sensors. P_{II} proteins were known to be localized in chloroplasts and to regulate a key enzyme of arginine biosynthesis, NAGK, in response to 2-OG level (Uhrig et al., 2009). From biochemical analyses of *Synechococcus* and *Arabidopsis* P_{II} and NAGK proteins, it seemed that P_{II}-NAGK interaction is highly conserved from cyanobacteria to higher plants: P_{II} enhances the catalytic activity of arginine-feedback-inhibited NAGK when P_{II} is ATP bound and not in complex with 2-OG and, conversely, ADP or 2-OG antagonizes productive interaction between P_{II} and NAGK (Beez et al., 2009; Mizuno et al., 2007b). From the conservation of critical amino acids in P_{II} and NAGK proteins involved in complex formation, we assumed that this interaction would be conserved in green algae, the phylogenetic predecessors of higher plants. Surprisingly, the *Chlamydomonas* P_{II} protein required glutamine in millimolar concentrations to interact with NAGK. Moreover, P_{II} proteins from the moss *Physcomitrella* and from the rice *Oryza* both showed a glutamine requirement similar to *Chlamydomonas* P_{II}. By contrast, *A. thaliana* P_{II} acted independent of glutamine. The slight truncation of the *At*P_{II} C-terminal extension turned out to be responsible for the divergent property.

To mechanistically explain why *At*P_{II} binds to NAGK independent of glutamine in contrast to the other P_{II} proteins requires an in-depth comparison of the structures. The C-terminal extension of *Cr*P_{II} forms a small helix-Q loop-helix structure, which wraps around the bound glutamine molecule. The bound glutamine acts like a pillar that puts the Q loop in place through its carboxyl group and holds the Q loop through main-chain interactions. Backbone interactions from the Q loop with Gln56 and Gly57 from the base of the T loop stabilize the highly flexible T loop and Q loop structures. In agreement, in crystals of *Cr*P_{II} not ligated to NAGK, these structures are disordered, which suggests that the flexible T loop and Q loop become structured upon NAGK binding. This agrees with the fact that NAGK arranges major contacts to the T loop of P_{II}. A *Cr*P_{II} variant with the C terminus truncated to the length of bacterial P_{II} proteins was unable to interact with NAGK, although it appeared to be an intact P_{II} protein with respect to ternary structure and 2-OG

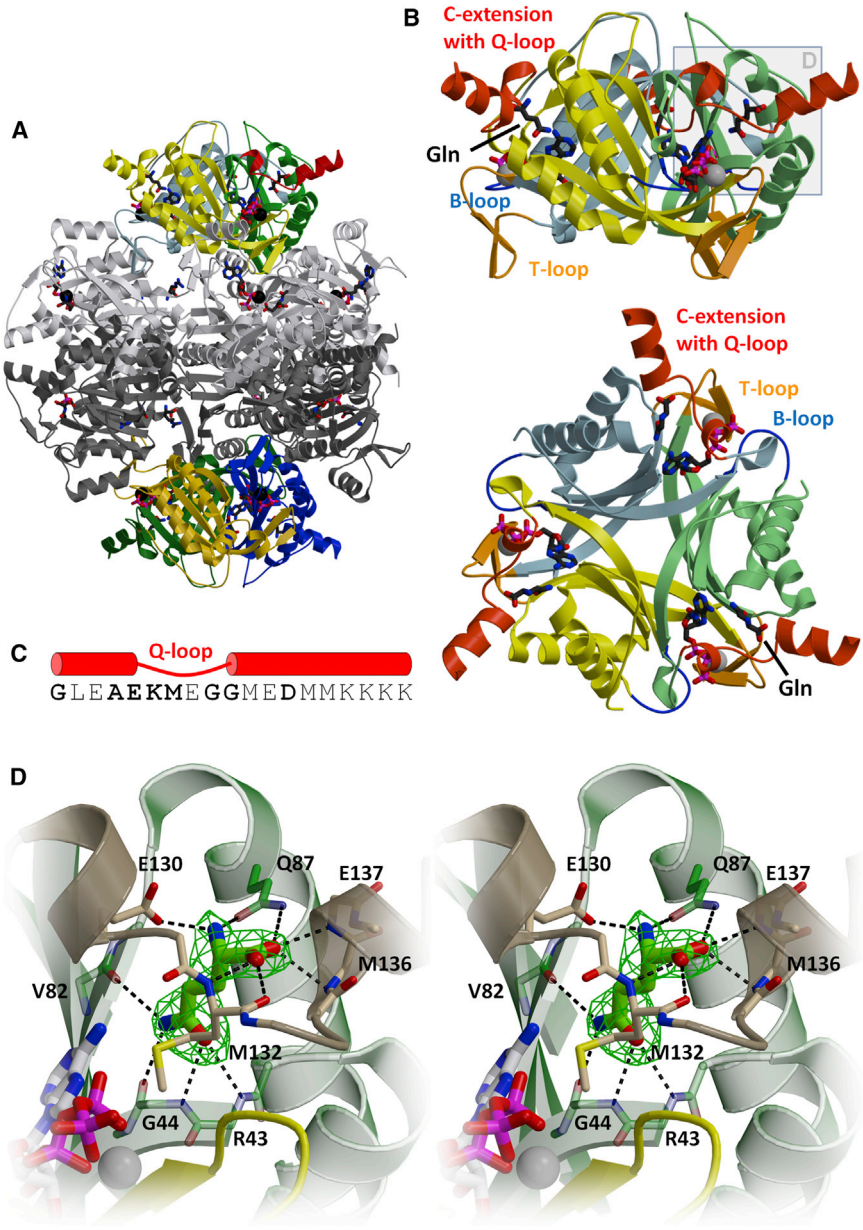

Figure 5. Glutamine-Bound *Cr*P_{II} in Complex with *At*NAGK

(A) P_{II} monomers are individually colored; NAGK is in two shades of gray. In the P_{II} trimer on top, one C-terminal extension is highlighted in red.

(B) Side and top views of the P_{II} trimer from the complex. The T loop, B loop, and whole C-terminal extension are individually colored. The whole C-terminal extension, consisting of two α helices connected by the Q loop, constitutes a large portion of the glutamine-binding site.

(C) The sequence of the C-terminal extension, highlighting the consensus motif.

(D) A stereo close-up of the glutamine-binding site reveals a sophisticated hydrogen-bonding network. Electron density for glutamine is shown as an $F_O - F_C$ omit map contoured at 4σ. Data collection and refinement statistics of the *Cr*P_{II}-*At*NAGK complex are shown in Table S1.

binding. This suggests that the T loop in the absence of the Q loop cannot adopt the structure that binds NAGK. The interactions between the Q loop and the T loop are apparently necessary to stabilize the structure. The same stabilizing interactions between the C terminus and the T loop are found in the *A. thaliana* P_{II}-NAGK complex (see Figure 6B). However, in this case, no binding of glutamine is required. Owing to the deletion of three amino acids at the Q loop, an aspartate residue (Asp129) projects into the glutamine-binding pocket and a hydrogen-bonding network with water molecules fills the cavity. A key residue for determining the structure of the T loop in *Cr*P_{II} is Gln56 (corresponding to Gln50 in *At*P_{II} and Gln39 in *S. elongatus* P_{II}). The importance of this residue for stabilizing T loop conformations was originally recognized in *E. coli* P_{II} protein GlnB (Jiang et al., 1997). In bacterial P_{II} proteins, different Gln39 contacts switch the T loop in various states (Huergo et al., 2013; Zeth et al., 2014). In both *Cr*P_{II} and *At*P_{II} complexes with *At*NAGK, the corresponding glutamine side chain forms a bridge between the γ-phosphate oxygen of the bound ATP molecule and the backbone Met132 (*Cr*P_{II}) (Met126 in *At*P_{II}), thereby stabilizing the tightly flexed T loop conformation. As observed here for the *Cr*P_{II}-*At*NAGK structure, all P_{II} sites of the *A. thaliana* P_{II}-NAGK structure are occupied by ATP, although no ATP was added to the crystallization solution and must have been acquired from contaminating ATP in the ADP solution (Mizuno et al., 2007b). This demonstrates the strict ATP requirement for complex formation. This can be explained if we assume that the Gln56 interaction between ATP and the C-terminal backbone is necessary to fix the T loop in the flexed conformation necessary for insertion into the crevice of NAGK. In cyanobacteria, the C-terminal Q loop extension does not exist but, nevertheless, P_{II} avidly interacts with NAGK. Moreover, this interaction does not require ATP, which indicates that in this case the T loop can fold into the NAGK-fitting structure without engaging the Gln39-ATP contact. Indeed, in the *S. elongatus* P_{II}-NAGK structure, which is devoid of ATP, Gln39 is not involved in any contacts (Llácer et al., 2007).

Of 55 examined P_{II} C-terminal sequences of plants including green algae (Figure S4), only the 5 representatives of the *Brassicaceae* family have the deletion found in *Arabidopsis*, and of the 50 remaining cases, only 3 have significant alterations in the Q loop consensus motif. From this almost invariant conservation, we deduced that the formation of the P_{II}-NAGK complex in

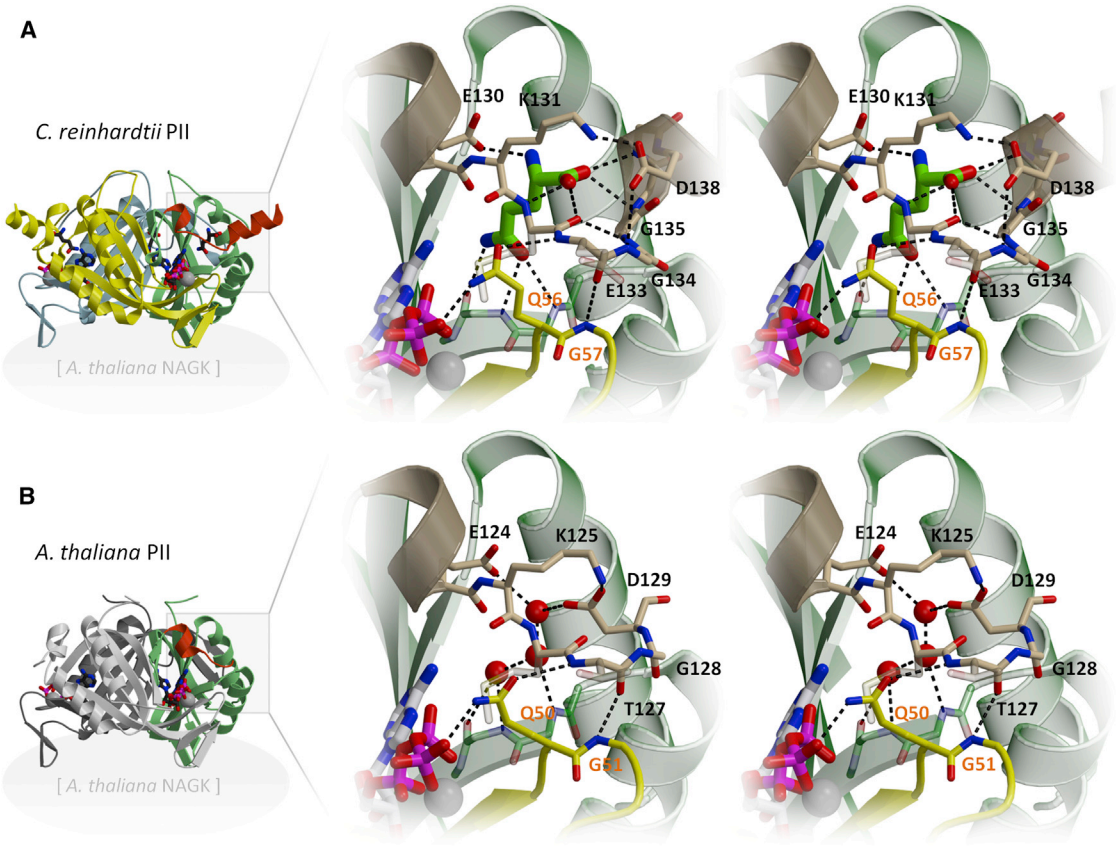

Figure 6. Detailed View of the Interactions between the Q Loop and T Loop in P_{II} from *C. reinhardtii* and *A. thaliana*

(A and B) *C. reinhardtii* (A) and *A. thaliana* (B). In both complexes, the structured Q loop forms the same stabilizing hydrogen bonds with the γ-phosphate-coordinating glutamine in the T loop (Q56 in *Cr*P_{II}, Q50 in *At*P_{II}) and the subsequent glycine (G57/G51). Structurally, the twin glycine residues (G134 and G135 in *Cr*P_{II}) of the Q loop provide space for the glutamine ligand. Owing to the deletion of three amino acids, including the second glycine, the glutamine-binding site cannot form in *At*P_{II}, as the first glycine is directly followed by Asp129. With the involvement of three bridging water molecules mimicking the bound ligand, this stunted Q loop can fold independent of glutamine binding, whereas in *Cr*P_{II} the folding of the whole C extension depends on glutamine binding. The three water molecules in *At*P_{II} were identified in a reevaluation of the experimental data (see Extended Experimental Procedures).

(C) Sequence alignment of P_{II} C termini of plants and cyanobacteria, highlighting the Q loop consensus motif (bold) and the deletion of three amino acids in the *Brassicaceae* family. Color labeling shows secondary structure assignment (strands, cyan; helices, pink) according to the *Cr*P_{II}-*At*NAGK and *At*P_{II}-*At*NAGK complex structures. An extended alignment including 55 plant P_{II} sequences is shown in Figure S4.

plants generally requires binding of glutamine to the Q loop, and this was confirmed by examination of *Physcomitrella* and *Oryza* P_{II} proteins. The half-maximal effective concentration of glutamine was determined to be in the millimolar range, which likely reflects the K_d of the glutamine-binding site (EC_{50} of 2.4 mM, 9.2 mM, and 6.6 mM for *Chlamydomonas* P_{II}, *Physcomitrella* P_{II}, and *Oryza* P_{II}, respectively). Notably, these glutamine concentrations are within the range of the estimated concentrations (2.5–20 mM) of glutamine in different plant species such as tobacco, spinach, and barley (Fritz et al., 2006; Riens et al., 1991; Winter et al., 1993, 1994). Therefore, the low-affinity glutamine-binding mode by the P_{II} Q loop is perfectly tuned for sensing physiologically relevant glutamine levels, which strongly implies regulatory significance. Only when the glutamine concentration in the chloroplast is in the range of several millimolar could P_{II} efficiently activate NAGK and antagonize feedback

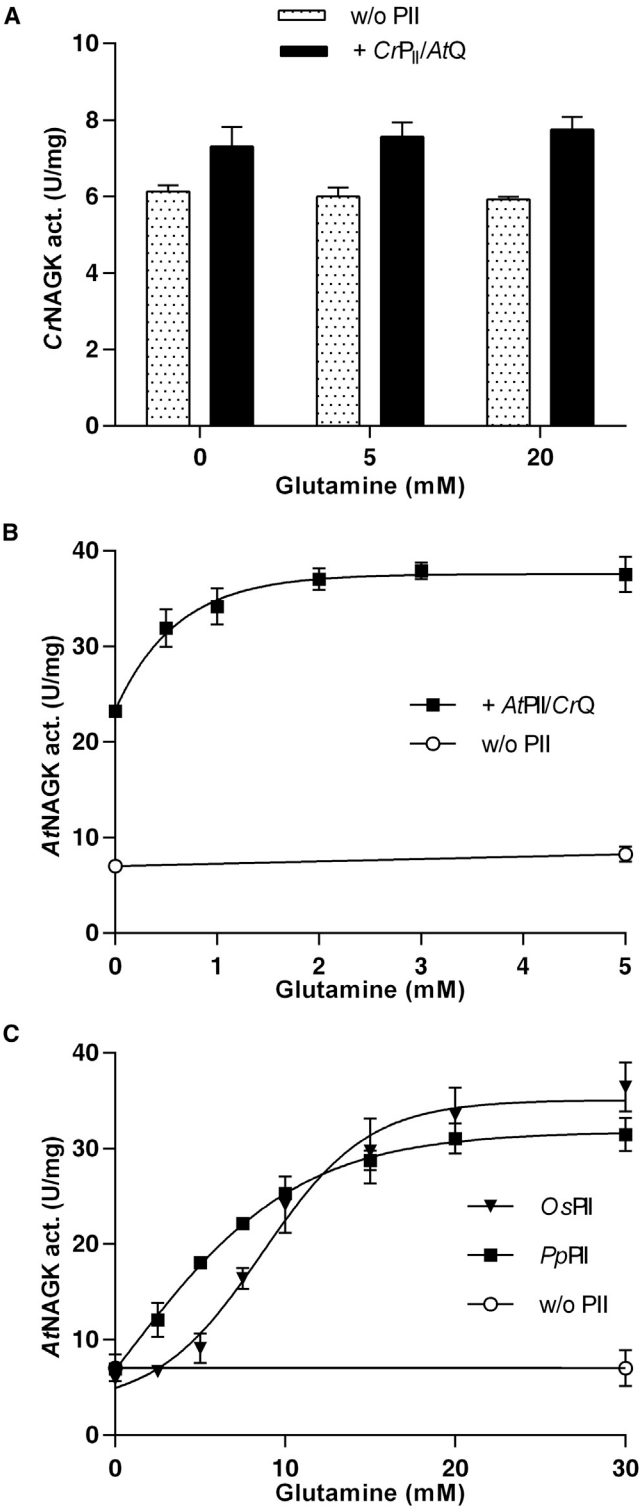

Figure 7. The C-Terminal Segment of P_{II} Proteins from Plants Other Than *A. thaliana* Mediates P_{II} Signaling toward NAGK

(A and B) Chimeric P_{II} proteins consisting of (A) *Cr*P_{II} with the C-terminal Q loop of *A. thaliana* (*Cr*P_{II}/*At*Q) and (B) *A. thaliana* P_{II} with the C-terminal Q loop of *C. reinhardtii* (*At*P_{II}/*Cr*Q) were tested for glutamine-dependent activation of cognate arginine-feedback-inhibited NAGK.

(A) *C. reinhardtii* NAGK with or without the *Cr*P_{II}/*At*Q chimera was assayed under standard conditions containing 400 μM arginine in the absence or presence of 5 or 20 mM glutamine.

(B) Glutamine-dependent activation of arginine-feedback-inhibited *At*NAGK by the *At*P_{II}/*Cr*Q chimera.

(C) Glutamine-dependent activation of arginine-feedback-inhibited *At*NAGK by *Physcomitrella* P_{II} (*Pp*P_{II}) or *Oryza* P_{II} (*Os*P_{II}).

Assays in (B) and (C) contained the NAGK feedback inhibitor arginine at 20 mM and increasing concentrations of glutamine, as indicated. The activity of NAGK in the absence of any P_{II} protein (without P_{II}) at 0 and 5 mM glutamine (B) and 0 and 30 mM glutamine (C) is indicated.

The standard deviation from triplicate measurements is indicated by error bars.

inhibition by arginine. Indeed, glutamine levels in plants strongly respond to nitrogen status, with amplitude changes of almost 200-fold reported in tobacco leaves (Fritz et al., 2006). In *Chlamydomonas* cells, glutamine levels are 10-fold lower under nitrogen deficiency (Bölling and Fiehn, 2005), accompanied by a similar decline in metabolites of the ornithine/arginine synthesis pathway. Thus, the flow of amino acids into this pathway, which feeds into the arginine pool as well as into polyamines, appears to be controlled by the glutamine level via the P_{II} protein. Arginine has multiple metabolic roles in addition to its roles as a building block in protein biosynthesis, and can also be used as a nitrogen-storage molecule (Llácer et al., 2008). Only at high glutamine levels, which indicate sufficient ammonia supply, is synthesis of nitrogen-storage compounds beneficial.

An intriguing question concerns the evolution of *Brassicaceae* P_{II} proteins. A three amino acid deletion at the Q loop has apparently occurred to generate a glutamine-insensitive variant. Grafting the Q loop from *Chlamydomonas* onto the *A. thaliana* P_{II} body restored glutamine sensitivity at least partially, and shows that *A. thaliana* P_{II} can be converted back to a glutamine-sensing protein. By contrast, the *A. thaliana* C terminus transplanted onto the *Cr*P_{II} body was almost nonfunctional, which indicates that additional modifications in the P_{II} body are required to evolve a functional glutamine-insensitive P_{II} protein. Although glutamine sensing is lost in the *Brassicaceae*, their P_{II} protein is still responsible for the regulation of the ornithine/arginine synthesis pathway; accordingly, P_{II} knockdown lines of *A. thaliana* show impaired accumulation of metabolites from the ornithine/arginine pathway (Ferrario-Méry et al., 2006). The selective pressure that resulted in glutamine-insensitive *Brassicaceae* P_{II} proteins is unknown, but it could point toward a special feature of nitrogen metabolism in this plant family.

Glutamine is the primary nitrogen status reporter in many bacteria (Forchhammer, 2007). There, information about the cellular glutamine status is perceived by the P_{II} signaling system via glutamine-sensitive modification enzymes, such as the uridylyltransferase/uridylyl-removing enzyme in *E. coli* (Adler et al., 1975; Jiang and Ninfa, 2011). In the development of green algal chloroplasts from cyanobacterial ancestors, evolution has reinvented glutamine sensing by P_{II} signal transducers using a modular concept: a small extension was attached to the C terminus, which controls the interaction of P_{II} with receptors through a subtle network of interprotein interactions for which binding of glutamine is necessary. This is a striking example of convergent

evolution in one of nature's most abundant signaling proteins. By the identification of P_{II} as a glutamine sensor, this work opens the way to a deeper understanding of nitrogen homeostasis in plants and may help optimize plant breeding for crop yield improvement.

EXPERIMENTAL PROCEDURES

Detailed methods are given in Extended Experimental Procedures.

Cloning, Expression, and Purification of P_{II} Proteins

Recombinant P_{II} from *A. thaliana* (*At*P_{II}) and from *C. reinhardtii* P_{II} (*Cr*P_{II}) with a C-terminally fused Strep-tag II sequence were overexpressed and purified as described previously (Beez et al., 2009; Ermilova et al., 2013). The *Cr*P_{II}-ΔC-coding gene was constructed using standard PCR techniques. *At*P_{II}/*Cr*Q chimera, *P. patens Pp*P_{II}, and *O. sativa Os*P_{II} genes were commercially synthesized. All genes were cloned into the Strep-tag fusion vector pASK-IBA3plus. Overexpression and purification of the P_{II} proteins by affinity chromatography on Strep-Tactin columns were described previously (Heinrich et al., 2004). Biochemical properties of the purified *Cr*P_{II} and *Cr*P_{II}-ΔC proteins are shown in Figure S3.

Cloning, Expression, and Purification of NAGK Proteins

Recombinant *At*NAGK was overexpressed and purified as described previously (Beez et al., 2009). For recombinant *Cr*NAGK, a gene corresponding to mature chloroplast-localized *Cr*NAGK was commercially synthesized, cloned into vector pET15b, and purified as recombinant *At*NAGK. Analysis of purified *Cr*NAGK and *At*NAGK by gel filtration is shown in Figure S2. A multiple sequence alignment of plant and cyanobacterial NAGK proteins, including predicted chloroplast transit peptides for *Cr*NAGK and *At*NAGK, is shown in Figure S1.

Surface Plasmon Resonance Analysis

SPR experiments were performed using a Biacore X biosensor system at 25°C in HEPES-buffered saline (HBS buffer) as described previously (Maheswaran et al., 2004). His_6-NAGK was immobilized on flow cell (FC) 2 to a density of approximately 3,000 resonance units (RUs). The analyte solutions contained P_{II} proteins (100 nM) in HBS buffer and were incubated with various effector molecules on ice for 5 min and then injected (50 μl) into FC1 (control) and FC2 of the sensor chip. The specific binding of P_{II} to NAGK was recorded as the response signal difference FC2 − FC1 (ΔRU).

Isothermal Titration Calorimetry

ITC experiments were performed on a MicroCal VP-ITC instrument in 10 mM potassium phosphate (pH 7.5), 100 mM NaCl, and 2 mM $MgCl_2$ at 20°C.

Enzymatic Assay for NAGK Activity

A coupled enzyme assay was used to determine NAGK activity in which the production of ADP was coupled to the oxidation of NADH (Beez et al., 2009). The reaction mixture contained 2.4 μg of P_{II} protein; the reaction was started by adding 3 μg of NAGK. Means of triplicate experimental determinations are shown. From the velocity slopes, the catalytic constants were calculated using the GraphPad Prism 6.01 software program.

Crystallization, Sample Preparation, Data Collection, and Structure Determination

Crystallization trials were performed in a standard vapor-diffusion setup. Crystals of the *Cr*P_{II} apo form grew in a solution containing 3 mg/ml *Cr*P_{II} in 10 mM Tris (pH 7.5), 100 mM NaCl, 2 mM ATP, 2 mM $MgCl_2$, 5 mM Gln, and 10% (v/v) glycerol and a reservoir solution containing 0.15 M $(NH_4)_2SO_4$, 0.1 M HEPES (pH 7.0), and 20% (w/v) PEG 4000. For the preparation of the *Cr*P_{II}•Mg-ATP•2-OG complex, these crystals were soaked in a droplet of reservoir solution supplemented with 10 mM ATP, 10 mM $MgCl_2$, 10 mM 2-OG, and 20% (v/v) glycerol. Crystals of the *Cr*P_{II}-*At*NAGK complex grew in a solution containing 2 mg/ml protein (with a 1:2 molar ratio of *Cr*P_{II}:*At*NAGK) in 10 mM Tris (pH 7.8), 100 mM NaCl, 40 mM Arg, 10 mM Gln, 2 mM $MgCl_2$, 2 mM ADP, 10 mM NAG, and 5% (v/v) glycerol and a reservoir solution containing 200 mM NaCl, 100 mM Na/K phosphate (pH 6.2), and 50% (v/v) PEG 200. Data were collected under cryogenic conditions.

The *Cr*P_{II} structure was solved by molecular replacement using the *Se*P_{II} structure 2XZW. The *Cr*P_{II}-*At*NAGK complex structure was solved on the basis of the *At*P_{II}-*At*NAGK structure 2RD5. Data processing and refinement statistics are summarized in Table S1.

ACCESSION NUMBERS

The Protein Data Bank ID codes for the structures reported herein are 4USH (*Cr*P_{II} apo), 4USI (*Cr*P_{II} with ligands), and 4USJ (*Cr*P_{II}-*At*NAGK complex).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.10.015.

AUTHOR CONTRIBUTIONS

V.-R.C. biochemically analyzed *Cr*P_{II}/*Cr*NAGK, carried out the crystallization and diffraction experiments, and solved and analyzed the crystal structures; E.E. evaluated results of the *Cr*P_{II}/*At*NAGK experiments; T.L. carried out the *Cr*P_{II}/*At*NAGK experiments; J.L. designed and performed the cloning experiments; E.M. prepared and tested the *Os*P_{II} and *Pp*P_{II} proteins; C.H. purified the proteins and performed enzyme assays; M.D.H. analyzed sequences, solved and analyzed the crystal structures, and wrote the manuscript; and K.F. designed, analyzed, and evaluated the experiments and wrote the manuscript.

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